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THE HIGH PERFORMANCE MOLECULAR EXCLUSION CHROMATOGRAPHY OF PGBX--ETC(U)
MAY 79 H W SHMUKLER, E SOFFER, S F KWONG

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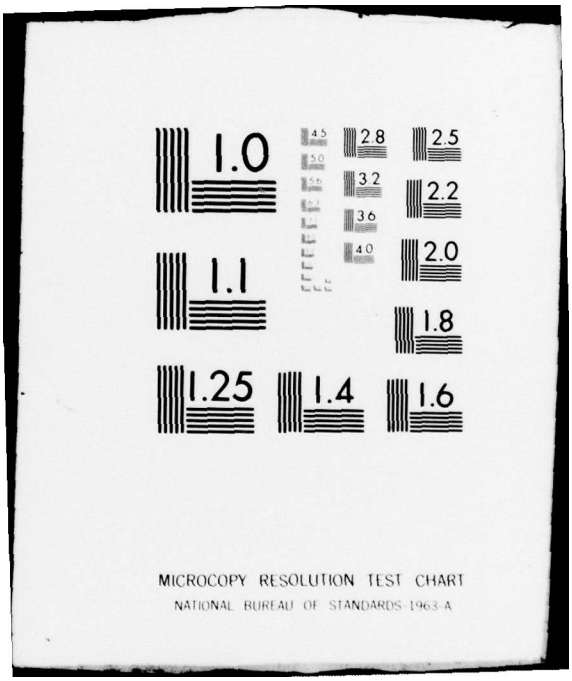
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(14) REPORT NO. NADC-79085-60

(12) LEVEL II

(6) THE HIGH PERFORMANCE MOLECULAR EXCLUSION CHROMATOGRAPHY OF PGB$_x$.

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(11) 1 May 1979

(12) 9p.

(9) Phase Report,

(17) ~~AIRTASK NO.~~ RF51524003
Work Unit No. SJ201

(16) F51524

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Prepared for
OFFICE OF NAVAL RESEARCH
Department of the Navy
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1. REPORT NUMBER NADC-79085-60	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) The High Performance Molecular Exclusion Chromatography of PGB_x		5. TYPE OF REPORT & PERIOD COVERED Phase Report 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) H. W. Shmukler, E. Soffer, S.F. Kwong		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Aircraft & Crew Systems Technology Directorate Naval Air Development Center Warminster, PA 18974		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS AIRTASK NO. RF51524003 Work Unit No. SJ201
11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research Department of the Navy Arlington, VA 22217		12. REPORT DATE 1 May 1979 13. NUMBER OF PAGES 7
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) 15a. DECLASSIFICATION/ DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

PGB_x
High Performance
Molecular Exclusion
Chromatography
prostaglandin derivative

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report describes a high performance molecular exclusion chromatographic (HPMEC) separation of ~~PGB_x~~ a prostaglandin derivative that yields information concerning the distribution of components according to molecular size. It should be useful in describing the homogeneity of ~~PGB_x~~ a prostaglandin derivative preparations.

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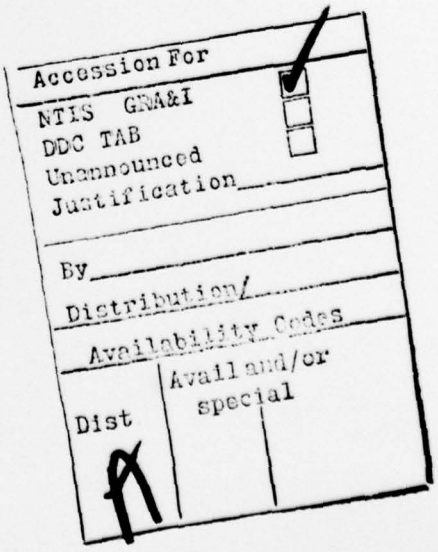


INTRODUCTION

In 1973, Polis et al[1] first synthesized PGB_x from 15-diketo PGB_1 under rigidly controlled conditions of alkalinity, temperature and time. Even under these controlled conditions, the PGB_x product that was isolated was found to be a complex mixture of polymers of varying molecular size and configurations. Because of the importance of PGB_x in the reversal of the effects of myocardial ischemia[2] and cerebral ischemia[3] in animals and its possible use in humans, it was deemed necessary to purify PGB_x to avoid possible side effects from contaminants. Studies have been under way in this laboratory to separate the active component from the PGB_x complex. At the present time, the purification of PGB_x is followed by the in vitro assay, which is based on the recovery of oxidative phosphorylation upon the addition of PGB_x to aged degraded rat liver mitochondria[4]. Unfortunately, this type assay yields no information concerning the homogeneity of the PGB_x preparations.

EXPERIMENTAL

PGB_x was prepared according to Polis et al[4] and dissolved as the sodium salt in the carrier buffer. The concentration was 1.0 mg/ml.

High performance molecular exclusion chromatography (HPMEC) was carried out with the Hewlett-Packard Liquid Chromatograph Model 1084A (Avondale, PA). The ME column was 4.6 mm I.D. X 250 mm long packed with Lichrospher S1-300 (Brownlee Laboratories, Santa Clara, CA). The first column used in this study eventually became clogged with sample and buffer impurities to the point where solvent flow stopped. To overcome this, a guard column (2 mm I.D. X 100 mm long) packed with Controlled Pore Glass, pore size 1250Å (Corning Glass Co., Corning, NY) was then placed between the injector and the S1-300 column in order to prevent clogging of the column packing.

Chromatography was carried out isocratically in 0.05 M KH_2PO_4 + K_2HPO_4 buffer, containing 0.001 M NaN_3 as a preservative to prevent mold growth. The pH of the carrier buffer was adjusted by varying the mixtures of two buffers stored in the solvent delivery reservoirs. Reservoir A contained pH 6.4 buffer and reservoir B contained pH 7.8. The pH of the buffer mixture was determined by measuring the pH of the solvent stream emerging from the detector. The separation was followed with a UV detector (λ254 nm) and a refractive index monitor in series. The following instrumental parameters were used for the chromatography: flow-rate, 1.0 ml per minute; column pressure, 140 bar; column temperature, 30°; chart speed, 1.0 cm per minute; UV detector, zero setting, 10 percent; UV detector attenuation, 64 x 10^{-4} AU per cm. When an RI detector was used (LDC Refracto-Monitor, LDC Corp., Riviera Beach, FL) it was set at attenuation "8" and connected to a Hewlett-Packard Integrator set at attenuation "16."

RESULTS AND DISCUSSION

Previously, it was reported from this laboratory[5] that the PGB_x complex could be separated by gel filtration on Sephadex G-100 or G-150 (Pharmacia Inc., Piscataway, NJ) using aqueous neutral phosphate buffers as carrier solvents. Unfortunately, these Sephadex packings compress under pressure making it mandatory that solvents be delivered through the column by gravity. This packing shortcoming results in excessive and nonreproducible retention times. For this reason, this type of gel filtration is only practical for preparative applications. In order to apply the gel filtration principle to analytical chromatography, studies were undertaken to evaluate commercial molecular exclusion packings suitable for high performance liquid chromatography[5].

Recently the Lichrospher family of highly porous silica spheres became available commercially in packed columns. These were evaluated and found suitable for the analysis of PGB_x preparations in terms of molecular size. According to the manufacturer's specifications, Si-300 has an exclusion limit for polystyrenes between 150,000 and 300,000 when chromatographed in chloroform. Since no information was available as to the exclusion limits in aqueous carrier solvents, the Si-300 column was calibrated with known standards in 0.05 M, pH 6.85 buffer. These results are listed in table I.

Table I

Calibration of Aqueous MEC on Lichrospher Si-300

Standard	M.W.	R.T.(minutes)
Dextran Blue 2000*	2×10^6	2.7
Stachyose	666.6	6.0
Adenylic Acid	347.23	5.7
EDTA**	292.24	5.7
Sucrose	342.30	5.8
KCl	74.55	5.9
PGB_x		2.8 4.2

*Pharmacia, Inc., Piscataway, NJ

**N,N'-1,2-ethane diylbis (N-(carboxy-methyl)glycine)

Unfortunately, water soluble standards in the range of 1000 - 5000 daltons are not available so that the column calibration is not complete. Nevertheless, the data for Dextran Blue 2000 indicates the retention time of large

molecular weight substances, while the other standards define the retention time of small molecules; therefore this analytical system can separate only the large molecules from the small molecules. The retention time of PGB_x shown in table I indicates the separation of two components, one emerging after 2.8 minutes that exhibits a large molecular weight and the other emerging after 4.2 minutes suggesting an intermediate molecular weight between the completely excluded standard and the nonexcluded standard.

In order to determine the optimum pH for the MEC separation of PGB_x, the pH was varied by changing the "percent B" command of the solvent delivery system. The column was first equilibrated with the new buffer, and then the pH of the solvent flow-to-waste was measured. The samples were then injected and MEC performed. Usually three chromatographic runs were made in order to be sure that the column was equilibrated with the carrier buffer. The results of these experiments are shown in figure 1. The pH ranges used in this study were limited on the acid side by the insolubility of PGB_x at low pH (below pH 6.2), and the chemical instability of PGB_x at alkaline pH (above pH 8.5), and the instability of the S1-300 packing at alkaline pH (pH 8.0). At pH 7.45, PGB_x chromatographs as a single component with a retention time equivalent to the lower molecular weight standards. From pH 7.0 down to pH 6.6, PGB_x chromatographed as a two-component molecular weight system. In addition, the areas of the two components changed as the pH was lowered. Figure 1 shows a progressive increase in the large molecular weight component with lowered pH, while the area of the small molecular weight component decreases.

In a previous study[5], it was reported that gel filtration of PGB_x on Sephadex packings using methanol as a carrier solvent yields only one chromatographic peak. Therefore, PGB_x MEC on S1-300 with methanol was also tried. Table II lists the retention time of molecular weight standards and PGB_x when chromatographed on S1-300 with methanol. These results show that PGB_x, when chromatographed in this system, emerges in the void volume suggesting that the molecular weight of the complex is above 5000.

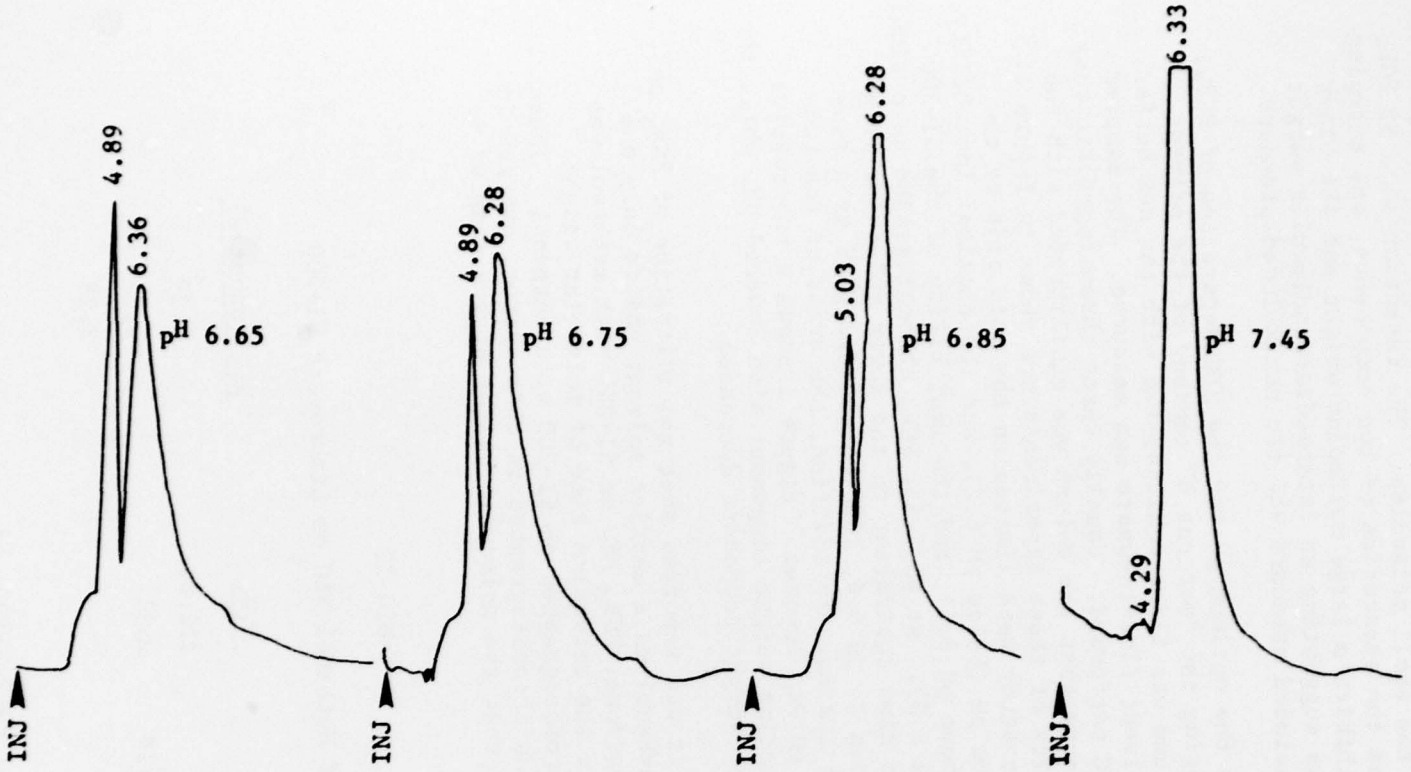
Table II

Calibration of Methanol MEC on Lichrosorb S1-300

Standard	M.W.	R.T.(minutes)
Diethyl pthalate	222.24	4.29
Polypropylene glycol*	4000	4.27
PGB_x		2.28

*Polysciences, Inc., Warrington, PA

The HPMEC of PGB_x in aqueous neutral phosphate buffer, described in this report, is similar to that reported for PGB_x gel filtration on



10 µg of PGB_x was injected and chromatography performed as described under "Experimental." The numbers at each peak indicate peak retention time in minutes. The time of sample injection is indicated on each chromatogram.

Figure 1 - THE EFFECT OF p^H ON HIGH PERFORMANCE MOLECULAR EXCLUSION CHROMATOGRAPHY OF PGB_x

Sephadex in a similar buffer system[5]. The advantage of the HPMEC over gel filtration is the use of a noncompressible packing. This type of packing is compatible with pressure delivery of the solvent, which in turn permits the chromatography to proceed at a predetermined constant flow-rate resulting in a rapid separation with reproducible retention times: two criteria for good analytical chromatography. With this method, it is now possible to determine the relative concentrations of high molecular and low molecular weight components in the PGB_x complex, thus giving some indication of its homogeneity.

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